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Neosporosis in Beagle dogs: Clinical signs, diagnosis, treatment, isolation and genetic characterization of *Neospora caninum*

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Abstract

Clinical neosporosis was diagnosed in a litter of five pups born to a Beagle bitch from Virginia, USA. Four of the pups developed limb weakness starting at 4 weeks of age. The dogs were suspected to have neosporosis based on clinical signs and empirically treated with Clindamycin (75 mg, oral, twice daily, total 150 mg) starting at 9 weeks of age and the dosage was doubled at 13 weeks of age. Antibodies to *Neospora caninum* were detected in sera of the dam and pups when first tested serologically at the age of 4 months. The owner donated the pup with the worst clinical signs and the dam for research; both dogs were euthanized. Viable *N. caninum* was isolated in gamma interferon gene knock out (KO) mice and in cell culture from the pup killed at 137 days of age. Tissue cysts, but no tachyzoites, were found in histological sections of brain and muscles. The isolate was also identified as *N. caninum* by PCR and sequence analysis and designated NC-9. *N. caninum* was neither isolated by bioassay in KO mice nor found in histological sections of tissues of the bitch. Clinical signs in the remaining three pups improved considerably after a 6-month treatment with Clindamycin; *N. caninum* antibody titers were still persistent in these pups at 23 months of age. Results indicate that medication with Clindamycin can improve clinical condition but not eliminate *N. caninum* infection.
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Keywords: Neosporosis; *Neospora caninum*; Clinical signs; Isolation; Mice; Gerbils; Cell culture; Bradyzoites; Sequence analysis

1. Introduction

The protozoan parasite *Neospora caninum* can cause a serious disease in dogs (Bjerkås et al., 1984; Dubey and Lindsay, 1996; Dubey, 2003) and the diagnosis has been verified by successful cultivation of the parasite from tissues or feces of a total of 20 dogs from Argentina, Australia, Brazil, Germany, United Kingdom, and the USA (Dubey et al., 2007). Although a breed predilection for clinical neosporosis in dogs is unknown, hunting breeds appeared to be affected more than other breeds. Previous reports of the isolation of *N. caninum* from tissues of clinically affected dogs were in a Boxer from England (Barber et al., 1995), a Collie from Brazil (Gondim et al., 2001), a Kleiner Munsterländer from Germany (Peters et al., 2000), a West Highland White Terrier from Australia (McInnes et al., 2006), and 10 dogs from USA including a English Springer Spaniel (Cuddon et al., 1992), a Rhodesian ridge back (Marsh et al., 1998), and eight Labradors (Dubey et al., 1988a,b, 2004; Hay et al., 1990). Additionally, *N. caninum* oocysts were found in feces of

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a Rottweiler from Argentina (Basso et al., 2001) and from the feces of five dogs, including a Dutch Shepherd, a Wire-haired Vizsla, a Hovawart, a German Spitz, and a Wire-haired Dachshund from Germany (Schares et al., 2005). We report the first confirmed neosporosis in a litter of Beagle dogs and report the biologic and genetic characterization of *N. caninum* isolate from one of the dogs.

2. Materials and methods

2.1. Naturally infected dogs

A 2-year-old abandoned Beagle bitch was found in Chase City, VA 23924 (latitude: 36.7715; longitude: 78.4254) in November 2004. The dog gave birth to five pups (nos. 1–5) on January 15, 2005 (Table 1). The pups appeared to be clinically normal until 4 weeks of age. Four (nos. 1–4) of the five pups eventually developed weakness in their limbs. Pup no. 1 (Missy) was a runt of the litter and developed clinical signs earlier than littermates. She never walked normally. At first, pup no. 1 had a limp in one of her rear limbs. At 4 weeks she was dragging her rear limbs that were limp and not stiff. At 6 weeks of age her limp leg became stiff overnight and was pointed forward. She had atrophy of the hindquarter of body and some knuckling under of the worst foot. At 10 weeks of age the dog learned to move around on two or three limbs. On occasion, she used her good rear leg to propel herself with a hopping motion. The dog was able to sit and used her forelimbs to hold herself.

Pup no. 2 developed limpness in rear legs at 6 weeks of age and rear limbs became stiff and were pointed forward at 8 weeks of age. The pup could move its rear legs independent of each other.

Pup no. 3 was able to sit up and was taking some steps at 6 weeks of age. At 8 weeks, one of his rear limbs had stiffened noticeably, the other rear limb was limp but not stiff. The rear limbs were pointed forward at 9 weeks of age. His condition continued to deteriorate. The owner donated this dog for research.

Pup no. 4 developed only mild clinical signs and had a hopping awkward limb motion.

Pup no. 5 walked normally at 4 weeks of age and never had any limb weakness or neural signs.

The pups were first examined on March 22, 2005 by a veterinarian at the SouthPaws Veterinary Referel Center, Springfield, VA when dogs were 9-week old. Three days later the pups were examined by a neurologist at the Hybla Veterinary Hospital, Alexandria, VA. At the time of their visit to veterinary hospital pups 1–4 weighed 2.5–4.0 kg and their weight had doubled a month later. Pups 1–4 were suspected to have neosporosis based on clinical signs and were medicated with Clindamycin (Antirobe, 75 mg, oral, twice daily, total 150 mg) starting at 10 weeks of age and the dosage was doubled at 13 weeks of age. Pup 5 was not medicated. Pups 1, 2, and 4 were continuously medicated with Clindamycin until they were 8-month old. Their clinical signs were improved except the stiffness of the limbs.

The owner contacted one of us (JPD) after search on the Internet about canine neosporosis. The pups were brought to the Animal Parasitic Diseases Laboratory (APDL), Beltsville on May 18, 2005 (Table 1) for consultation and a blood sample was obtained from the five pups. The owner donated the bitch and one of the worst affected pups (no. 3) for research because of poor prognosis in the pup and because the owner did not want to keep the infected bitch. The pup and the bitch were

Table 1
Antibodies to *Neospora caninum* in sera of the naturally infected bitch and her pups

Dog I.D.	Antibody responses							
	May 18, 2005				December 18, 2006			
	NAT	IFAT	ELISA		NAT	IFAT	ELISA	
			1:250	1:1000			1:250	1:1000
Pup 1 (Missy, female)	1600	800	2.404	2.361	800	800	0.580	0.755
Pup 2 (Biscuit, male)	400	400	0.687	0.484	400	200	0.605	0.707
Pup 3 (BB, male)[a]	800	800	0.255	0.207		Killed		
Pup 4 (Fellah, male)	800	800	0.430	0.316	800	400	0.716	0.166
Pup 5 (Rascal, male)	<25	50	0.255	0.201	Not bled			
Bitch (Sally)[b]	400	800	0.558	0.443		Killed		

[a] Euthanized 6/1/2005, NAT 1:200, IFAT 1:200, ELISA 0.605 (1:250), 0.707 (1:1000).
[b] Euthanized 6/8/2005, NAT 1:200, IFAT 1:400, ELISA 0.605 (1:250), 0.707 (1:1000).

euthanized on June 1, and June 8, 2005, respectively at the APDL. A blood sample was drawn on December 18, 2006 (23 months of age) from the three pups that were medicated with Clindamycin.

2.2. Serological examination

Sera from dogs were examined for antibodies to *N. caninum* using the indirect fluorescent antibody test (IFAT, Dubey et al., 1988b), direct *Neospora* agglutination test (NAT, Romand et al., 1998) and a recombinant antigen (NcGRA6) ELISA as described (Jenkins et al., 2005). For IFAT and NAT, sera were diluted two-fold starting at 1:25 dilution. For ELISA, sera were diluted 1:250 and 1:1000 and tested in triplicate at each dilution. Positive and negative control dog sera were included in each assay. A serum was considered positive if the mean O.D. reading was greater than the mean of the negative control sample plus 3S.D.

Sera from dogs were also examined for antibodies to *Toxoplasma gondii* using the modified agglutination test (MAT; Dubey and Desmonts, 1987) using a cut-off value of 1:25.

2.3. Necropsy examination of dogs and collection of materials for bioassays

Complete necropsy examinations were performed by one of us (JPD) on both dogs. The entire brain and spinal cords were removed. Specimens of cerebrum, cerebellum, pons, medulla, cervical, thoracic, and lumbar spinal cords, tongue, heart, lung, liver, adrenals, spleen, kidneys, eyes, sciatic nerve, oesophagus, stomach, intestines, and muscles from masseter, diaphragm, and limbs were fixed in buffered neutral 10% formalin. For direct microscopic examination, 2–3 mm pieces of the cerebrum of dogs were crushed on glass slides under cover slip, and examined microscopically for tissue cysts.

2.4. Attempted isolation of N. caninum from dogs in cell culture and rodents

For isolation of *N. caninum* in cell culture, brain, spinal cord, and muscle tissues were homogenized in saline, digested in trypsin or pepsin solutions (Dubey and Schares, 2006), and the mixture was incubated for 30 min at 37 °C, centrifuged, washed three times with Hanks balanced salt solution (HBSS), and then inoculated on to M617 or CV-1 cells in tissue culture flasks or rodents as described by Dubey et al. (1998, 2004). After 1 h incubation, the inoculum was removed, rinsed with plain medium, and fresh growth medium. Interferon gamma gene knock out (KO) mice and gerbils (Dubey et al., 1998, 2004) were inoculated with canine tissues (Table 2). To isolate *N. caninum* from single tissue cysts, microscopically identified tissue cysts in smears were removed, incubated for 5 min in trypsin to release bradyzoites, and after washing were inoculated subcutaneously in to KO mice (Table 2).

Rodents inoculated with canine tissues were examined for *N. caninum* and *T. gondii* parasites. Impression smears of lungs and brain of mice that died were fixed in methanol and examined after staining with Giemsa. Survivors were bled 6–8 weeks post inoculation (p.i.) and 1:25 dilutions of their sera were examined for *T. gondii* antibodies by MAT and *N. caninum* antibodies by NAT. Additional details are provided in Section 3.

2.5. Histological and immunohistochemical examinations

For histologic examination, paraffin embedded sections were cut at 5–6 μm thickness and examined

Table 2
Isolation of *N. caninum* from tissues of dog no. 3

Dog tissue inoculated	Animal species	No. inoculated	Outcome × days p.i.	*N. caninum* found
Pepsin-digested				
Brain	KO mice	5	D or DK 21, 26, 26, 26, 26	Yes
Spinal cord	KO mice	5	D 22, 23, 28, 29, 37	Yes
Muscle	KO mice	5	20, 28, 30, 30, 34	Yes
Brain and spinal cord	Gerbils	4	S 149	No+
Trypsin-digested				
Individual tissue cysts	KO mouse	1	23	Yes
	KO mouse	1	34	Yes
	KO mouse	1	S 162	No
	KO mouse	1	S 162	No

*D = died or killed when ill. S = survived, not ill. +Developed *N. caninum* antibodies (NAT 1:20 or higher).

after staining with hematoxylin and eosin (H and E). For immunohistochemical examination, a Dako Envision Peroxidase (Dako, Carpinteria, CA) rabbit kit and Envision system was used. Deparaffinized sections were stained with polyclonal rabbit anti-*N. caninum* and *T. gondii* antibodies (Lindsay and Dubey, 1989; Dubey et al., 2001, 2004), and polyclonal rabbit recombinant BAG (bradyzoite antigen)-1 antibodies (McAllister et al., 1996).

2.6. Molecular characterization

The *N. caninum* isolates in the present study were compared with the type strain (NC-1) of *N. caninum.* DNA was extracted from *N. caninum* infected tissues as described (Dubey and Sreekumar, 2003). PCR was performed with the *N. caninum*-specific ITS1 primers as described (Buxton et al., 1998). The PCR products were electrophoresed on an acrylamide gel and observed for the presence specific target fragment. The specific PCR product was cloned into the pCR2.1 vector (Invitrogen, Palo Alto, CA) and transformed into TOP10 cells (Invitrogen). Bacterial colonies containing the ITS-1 insert were cultured in LB/Ampicillin overnight, and plasmid DNA was purified using a Qiagen Mini prep kit (Qiagen, Valencia, CA). The insert from three clones was sequenced in both directions using the Big Dye terminator kit, version 3.1 (Applied Biosystems, Foster City, CA) using an automated ABI 3730xl sequencer. The resulting chromatograms were edited using Sequencher 4.7 software (Genecodes Corp., Ann Arbor, MI).

3. Results

3.1. Serologic results

By IFAT and ELISA, all pups and the dam had antibodies to *N. caninum* (Table 1). By NAT, four of the pups and the dam had antibodies to *N. caninum* (Table 1). The pup (no. 5) without clinical signs was negative by NAT, and had the lowest titer by IFAT when tested on May 18, 2005; this dog did not bleed on December18, 2006 because it was healthy; therefore it is uncertain if the pup was infected with *N. caninum* or only had colostral antibodies.

All pups were seronegative for *T. gondii* in 1:25 dilution of sera obtained in May 2005 as tested by the MAT. The dam had a *T. gondii* MAT titer of 1:50.

3.2. Necropsy and other results with Pup no. 3

3.2.1. Tissue cysts and bradyzoites

Tissue cysts were unevenly distributed in the cerebrum. In numerous squash preparations of samples of brain of the pup, some preparations had up to six

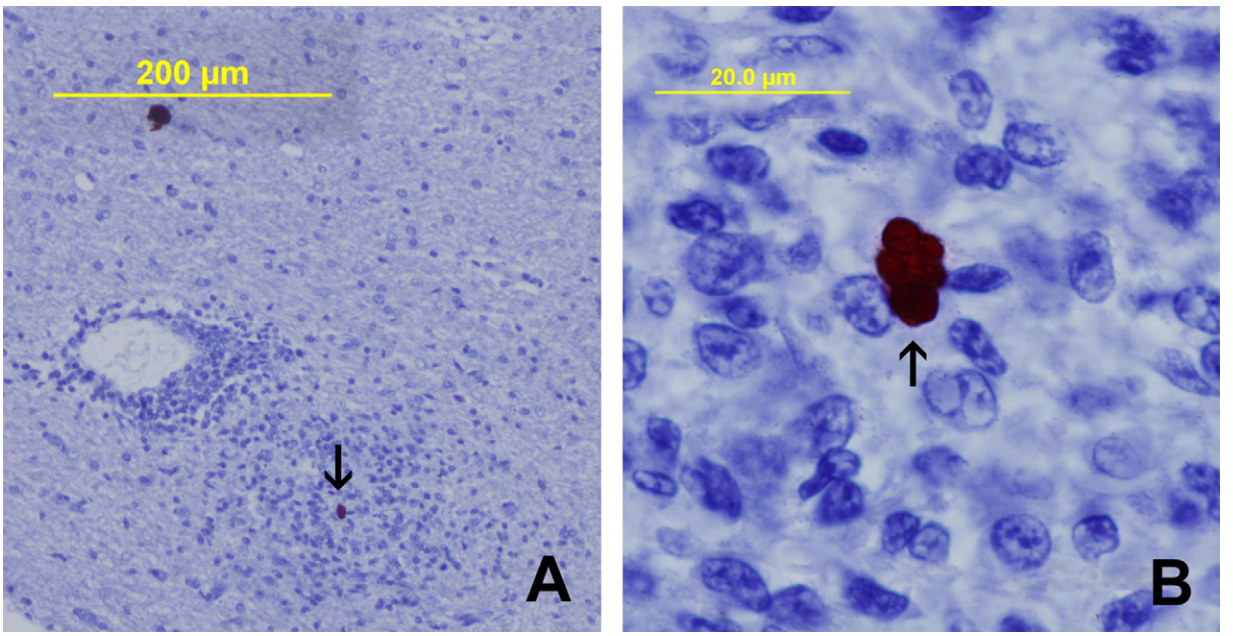

Fig. 1. Lesions and *N. caninum* in section of cerebrum of the pup. Immunohistochemical reaction with bradyzoite-specific antibodies. (A) Note perivascular infiltration of mononuclear cells and the presence of *N. caninum* in a microglial nodule (arrow). (B) A group of six or more bradyzoites (arrow) in the glial nodule.

tissue cysts whereas most had none. In total, 45 tissue cysts were found in 100 brain squashes of cerebrum. An intact tissue cyst and groups of individual zoites, that reacted intensely with BAG-1 antibodies, were found in sections of cerebrum (Fig. 1). The intact tissue cyst had no host reaction around it. Individual or groups of bradyzoites were surrounded by glial cells.

Infiltrations of mononuclear cells and occasional mineralization were present in all muscles examined (Fig. 2). Tissue cysts and individual bradyzoites were also seen in muscles from the right fore and hind limbs, left fore limb, and intercostals regions and these parasites reacted intensely to BAG 1 antibodies (Fig. 2).

3.2.2. *Isolation of N. caninum in rodents and cell cultures*

N. caninum was isolated by bioassays in KO mice inoculated with brain, spinal cord, muscles, and individual tissue cysts from the brain of the pup (Table 2). The KO mice inoculated with pup tissues died

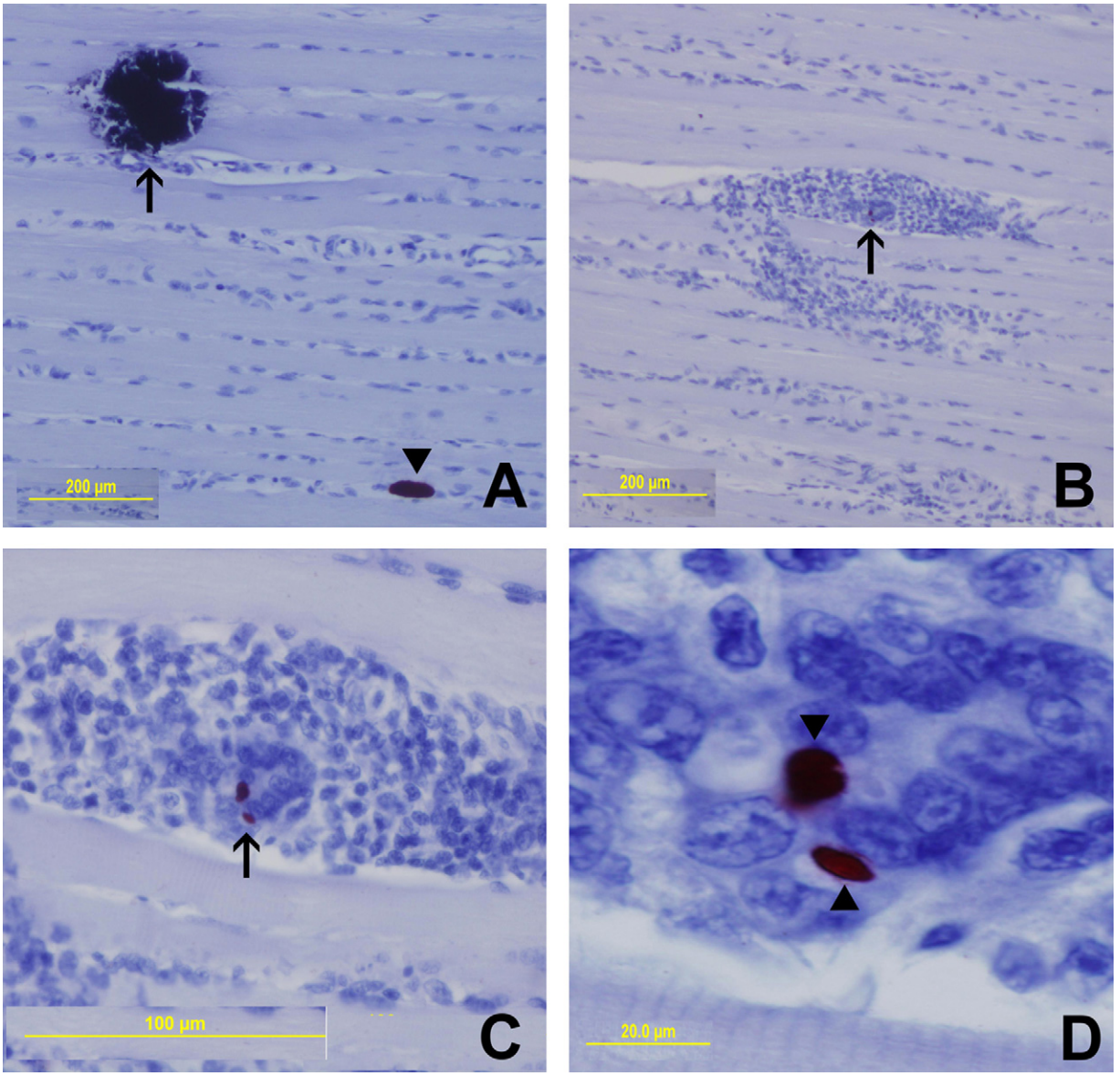

Fig. 2. Lesions and *N. caninum* in section of muscle from left fore limb of the pup. Immunohistochemical reaction with bradyzoite-specific antibodies. (A) Focal mineralization (arrow) and a tissue cyst (arrowhead). (B) An inflammatory focus (arrow). (C) Focal infiltration of mononucl; ear cells. Arrow points to bradyzoites. (D) Higher illumination of a single and a group of two bradyzoites (arrowheads).

from acute neosporosis; *N. caninum* tachyzoites were found in many organs. The gerbils inoculated with neural tissues of the pup-developed antibodies to *N. caninum* but tissue cysts were not seen when the gerbils were killed 3 months later.

N. caninum was neither seen histologically nor isolated from tissues of the bitch.

N. caninum was isolated in cell cultures inoculated with material derived from the pup. In cultures seeded with pepsin-digested tissues of the pup, tachyzoites were first seen in CV-1 cells 34 days after inoculation (culture no. 1) with brain homogenate and successfully passaged to new cells; the culture from the original flask was cryopreserved 78 days after seeding the culture. The M617 cells inoculated with the brain tissue and the CV-1 cells and M617 cells inoculated with spinal cord tissue became contaminated with bacteria and the flasks were discarded. *N. caninum* tachyzoites were seen in M617 cells 40 days after seeding with bradyzoites released by trypsinization of tissue cysts removed from squash preparations of the brain.

```
                0       10        20        30        40        50        60
NC9             TGAATCCCAAACAAAACATGAGCTTGTATCTCTCTCCTTCGGAGAGGGGTACATTCAAGA
CN1             ------------------------------------------------------------
BPA1            ------------------------------------------------------------
NC-SweB1        ------------------------------------------------------------
NC-Liverpool    ------------------------------------------------------------
NC-Nz1          ------------------------------------------------------------

                 . . . .70 . . . .80 . . . .90 . . . 100 . . . 110 . . . 120
NC9             AGCGTGATATACTACTCCCTGTGAGTTGTATCGCCTTCTTCATGTGGATATTTTGCACTA
CN1             ------------------------------------------------------------
BPA1            ------------------------------------------------------------
NC-SweB1        ------------------------------------------------------------
NC-Liverpool    ------------------------------------------------------------
NC-Nz1          ------------------------------------------------------------

                 . . . 130 . . . 140 . . . 150 . . . 160 . . . 170 . . . 180
NC9             CTTTTTTCAAGCGTTCTATTGAACGCCTGATAATGAAAGTGTGTGCATATATCCGGGAGT
CN1             ------------------------------------------------------------
BPA1            ------------------------------------------------------------
NC-SweB1        ------------------------------------------------------------
NC-Liverpool    ------------------------------------------------------------
NC-Nz1          ------------------------------------------------------------

                 . . . 190 . . . 200 . . . 210 . . . 220 . . . 230 . . . 240
NC9             GTACGGCGAAGGGACTCGGTCACTGGAAATTAATGTCTCTATTGGGACTTTAACTTCCAG
CN1             ------------------------------------------------------------
BPA1            ------------------------------------------------------------
NC-SweB1        ------------------------------------------------------------
NC-Liverpool    ------------------------------------------------------------
NC-Nz1          ------------------------------------------------------------

                 . . . 250 . . . 260 . . . 270 . . . 280 . . . 290 . . . 300
NC9             GAGTTTCTTCAATGTGCATTCTTTTTTCCCACACCGTTATTTTAAACAACAAATCTGGAT
CN1             ------------------------------------------------------------
BPA1            ------------------------------------------------------------
NC-SweB1        ------------------------------------------------------------
NC-Liverpool    ------------------------------------------------------------
NC-Nz1          ------------------------------------------------------------

                 . . . 310 . . . 320 . . . 330 . . . 340 . . . 350 . . . 360
NC9             AGCGTTTGAGGGAAGAGAAAGATGGTCTCTTTCTGTATTTCTCTCTATTCGCTTTCAGAT
CN1             ------------------------------------------------------------
BPA1            ------------------------------------------------------------
NC-SweB1        ------------------------------------------------------------
NC-Liverpool    ------------------------------------------------------------
NC-Nz1          ------------------------------------------------------------A--

                 . . . 370 . . . 380 . . . 390 . . . 400 . . . 410
NC9             TACTTACTAAAAACTATAATGTTTTTCTAAATTTTCAGCAATGGATGTCTT
CN1             ----------------------------------------------.....
BPA1            ----------------------------------------------.....
NC-SweB1        ----------------------------.......................
NC-Liverpool    ----------------------------.......................
NC-Nz1          A--------------------------------------------------
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Fig. 3. Alignment of the ITS-1 region from six different *N. caninum* isolates. Dashes denote nucleotide identity and dots denote missing nucleotides. Two nucleotide differences are observed between NC9 and NcNz1.

N. caninum was also propagated in cell cultures inoculated with lung homogenate of KO mice (Table 2) that were inoculated with tissues of the pup. For this, a KO mouse that became sick 21 days after inoculation with muscle digest was euthanized and its lung homogenate was inoculated onto M617 cells. Tachyzoites were seen in this culture 5 days later (culture no. 2) and this culture from the original flask was cryopreserved in liquid nitrogen 19 days after seeding the culture.

Another culture was made from a KO mouse that was inoculated with bradyzoites released from a single tissue cyst. For this, lung homogenate of a KO mouse that was euthanized on day 34 p.i. with bradyzoites released from a single tissue cyst was seeded onto M617 and CV-1 cells; tachyzoites were seen in both cultures and this culture (culture no. 3) was cryopreserved. These three cultures (from the brain homogenate, muscle homogenate, and from an isolated tissue cyst) were cryopreserved with ultimate objective to study the possibility of infections with mixed genotypes in the future.

3.2.3. Molecular studies

The identity of the isolate was confirmed as *N. caninum* by PCR. Amplification of the 411 bp fragment of the ITS-1 region was observed with all three isolates and positive control (NC-Original). No amplicons were observed in the negative control. The cloned fragments were sequenced and the sequences of the canine isolate were submitted to GenBank under accession number EF219139. All of the clones were identical to each other in sequence when compared to other *N. caninum* strains NC-9 ITS-1; the alignment revealed greater than 99% nucleotide agreement with *N. caninum* ITS1 sequences in GenBank''. Alignment of the representative ITS-1 sequences is shown in Fig. 3.

4. Discussion

4.1. Ante-mortem diagnosis

Ante-mortem diagnosis of neosporosis in animals is difficult. Clinical history, age of dogs, and serological examinations are helpful for the diagnosis of neosporosis in dogs. Most cases of canine neosporosis have been in congenitally infected dogs involving littermates (Dubey and Lindsay, 1996; Lindsay and Dubey, 2000). In most instances dogs are born without symptoms and begin to develop clinical signs 3 or more weeks after birth, and not all pups are affected to the same degree. In the present study, pups appeared normal at birth and developed clinical signs at 4 weeks of age. Paralysis of rear limbs, often with contracture, is the most consistent sign of neosporosis in dogs. Based on this knowledge the owner and the attending veterinarian correctly suspected neosporosis and started treatment and thus might have prevented further deterioration of the condition of the pups. Serological examination can help diagnosis of neonatal neosporosis in dogs. A negative antibody test at 1:25 dilution of serum in IFAT can exclude neosporosis. However, the magnitude of antibody titer is probably not important. Antibody titers have been generally low in cases of confirmed neonatal neosporosis (Dubey et al., 1998, 2004), even before treatment. In the present study, antibody titers were 1:200 or lower in pups but the pups had been given Clindamycin for 6 weeks before their sera were first tested for antibodies to *N. caninum*. Results of the present study indicate that antibody titers (and probably live parasites) persisted in pups that had been treated with Clindamycin for 6 months, the longest period of treatment reported to our knowledge.

4.2. Treatment

Treatment of clinical neosporosis in dogs is difficult and only partially effective (Hay et al., 1990). Treatment is most effective in early stages before muscular contracture has occurred. In the present study, bradyzoites were still present in the pup that had been medicated with Clindamycin for 8 weeks. Treatment with Clindamycin has been reported to improve clinical recovery in naturally infected dogs with neurological signs (Barber and Trees, 1996). However, rarely has there been an opportunity to verify parasiticidal effects in animals naturally infected with *N. caninum* (Dubey et al., 1995, 1998, 2005). In the present study, pup no. 3 had been treated with Clindamycin for approximately 2 months and had *N. caninum* tissue cysts but no demonstrable tachyzoites. Clindamycin affects multiplication of *N. caninum* tachyzoites but is thought to have little or no effect on bradyzoites (Lindsay et al., 1994). It is of interest that free isolated bradyzoites were seen in muscles and brain of pup; whether these bradyzoites had been released from tissue cysts or were newly formed could not be determined. In the related coccidium, *T. gondii,* reactivation of chronic infection in immunosppressed hosts is thought to occur when a tissue cysts rupture and released bradyzoites transform to tachyzoites and proliferate as tachyzoites; new bradyzoites and tissue cysts are formed from tachyzoites. There is some evidence, however, that bradyzoites can directly

transform into bradyzoites without transforming into tachyzoites first (Dubey, 2005). When and where tissue cysts rupture is unknown, immunosuppression by itself is not thought to cause cyst rupture. Tissue cysts rupture from time to time (and this event is rarely demonstrable histologically) and released bradyzoites are killed by an immune host, but begin to proliferate in an immunosuppressed host. The demonstration of bradyzoites in histological sections of tissues is difficult because of their small size, unless they are enclosed in intact cysts. The polyclonal rabbit serum used reacts with both tachyzoites and bradyzoites but anti-BAG1 rabbit serum reacts with only bradyzoites and not tachyzoites, however, it is not species specific because it reacts with bradyzoites of *N. caninum* (McAllister et al., 1996), *Besnoitia*, *Hammondia*, and probably other apicomplexans (Dubey and Sreekumar, 2003). The bradyzoites released from tissue cysts probably are responsible for maintaining cellular and humoral immunity in the host.

4.3. Isolation of N. caninum from dogs

Most isolates of *N. caninum* from dogs were from dogs with clinical signs (Dubey et al., 2007). In the present study we were able to isolate the parasite from the tissues of the pup with clinical signs but not from the asymptomatic bitch.

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